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(54) METHOD OF STABILIZING PROTEIN C OR ACTIVATED PROTEIN C AND STABILIZED COMPOSITION

METHODE ZUR STABILISIERUNG VON PROTEIN C ODER AKTIVIERTEM PROTEIN C UND STABILISIERTE VERBINDUNGEN

PROCEDE DE STABILISATION DE PROTéINE C ACTIVEE OU NON ACTIVEE ET COMPOSITION STABILISEE

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Descripti n

Technical Field of the Invention

[0001] The present invention relates to a method for stabilizing protein C or activated protein C which is derived from plasma or is prepared by using the genetic recombination technique. More particularly, the present invention relates to a method for stabilizing protein C or activated protein C when it is stored or subjected to procedures such as isolation and purification, lyophilization, treatment by heating, etc. and to a preparation stabilized by said method.

Technical Background

[0002] Protein C (hereinafter also referred to as "PC") is a kind of a vitamin K dependent protein, i.e. a protein containing γ-carboxyglutamic acid, and is activated to activated protein C (hereinafter also referred to as "APC") by thrombin in the presence of thrombomodulin present on the surface of the vascular endothelial cells. Activated protein C is a kind of a serine protease and exhibits a strong anti-coagulant activity by inactivating cofactors of the blood coagulation system such as Factor Va (FVa) and Factor VIIIa (FVIIIa). It is also known that activated protein C releases a plasminogen activator from the vascular wall to accelerate the fibrinolytic system. Furthermore, it is known that a defect in protein C causes severe thrombosis. Thus, it has been established that activated protein C is the most important factor which regulates blood coagulation and the fibrinolytic system. Therefore, protein C or activated protein C is expected to be exploited as a novel anti-coagulant or profibrinolytic agent.

[0003] It has been known that the amount of protein C present in a plasma or expressed in a tissue culture system is extremely low. Accordingly, in order to use protein C or activated protein C as an anti-coagulant or a profibrinolytic agent widely and safely, isolation and purification of protein C or activated protein C is important. In addition, storage as a solution or in frozen form for long periods of time, lyophilization or procedures for inactivation of contaminating viruses such as heating are indispensable to the process when protein C or activated protein C is industrially prepared on a large scale. However, storage, freezing or freeze-drying, or heat treatment of a highly pure protein C or activated protein C extremely lowers the activity thereof. There has also been no report on the stability of highly purified protein C or activated protein C. Under such circumstances, it is impossible to provide a highly pure protein C or activated protein C efficiently and stably on an industrial scale.

[0004] Under such circumstances, the present inventors have studied the stability of protein C or activated protein C, and as a result, have found that the activity of protein C or activated protein C can be maintained even after storage for a significant period of time or after procedures such as isolation and purification, lyophilization, heating, etc. by adding, to protein C or activated protein C, a salt buffer such as phosphate or citrate buffer containing sodium ions supplemented with at least one amino acid, and further adding either one or a combination of albumin and a non-ionic surfactant.

Disclosure of the Invention

[0005] The present invention relates to a method for stabilizing protein C or activated protein C which comprises adding, to a salt buffer containing protein C or activated protein C and sodium ions, at least one amino acid, and further either one or a combination of albumin and a non-ionic surfactant. More particularly, the present invention relates to a method for stabilizing protein C or activated protein C which comprises dissolving protein C or activated protein C in a salt buffer such as phosphate or citrate buffer containing sodium ions, and adding to said buffer at least one naturally occuring amino acid, e.g. glycine, alanine, lysine, arginine, aspartic acid, glutamic acid, etc. albumin and, in a preferred embodiment, an optional non-ionic surfactant, typically Tween 80. The invention also relates to preparations stabilized by said method.

Best Mode for Carrying out the Invention

[0006] Protein C or activated protein C used herein encompasses variants or derivatives thereof which substantially have APC activity and may be prepared by known methods, for example, by preparing protein C by isolation from human plasma or utilizing the genetic recombination technique and then activating protein C; by directly isolating APC from human blood; or by producing APC by using the genetic recombination technique, etc. Activation of protein C into APC can be carried out by any known method, for example, by activation with thrombin isolated from human or bovine blood, or by activation with an equivalent protease, etc.

[0007] Production of APC derived from blood can be conducted, for example, by activating protein C, which is purified from human plasma by affinity chromatography using an anti-protein C antibody, with human thrombin, and purifying the resulting activated protein C by cation chromatography (Blood, 63, p.115-121 (1984)); or by activating protein C

which is purified from human plasma by barium citrate adsorption and elution, fractionation with ammonium sulfate, DEAE-Sephadex column chromatography, dextran sulfate agarose chromatography and polyacrylamide gel electrophoresis, etc. to produce APC in accordance with the method described by Kisiel (J. Clin. Invest., 64, p.761-769 (1979)); or by activating a commercially available blood-coagulating preparation containing protein C to produce APC in accordance with the method described by Taylor et al. (J. Clin. Invest., 79, p.918-925 (1987)), and the like.

[0008] Production of APC utilizing the genetic recombination technique can be conducted, for example, in accordance with the methods described in Japanese Patent First Publication (Kokai) No. 61-205487, Japanese Patent First Publication (Kokai) No. 1-2338 or Japanese Patent First Publication (Kokai) No. 1-85084, etc. A process for preparing the starting material protein C or activated protein C as used herein is not limited to the above-mentioned procedures.

[0009] The thus prepared starting material protein C or activated protein C is isolated and purified by a combination of usual biochemical procedures for isolation and purification, including, for example, salting-out with ammonium sulfate, ion-exchange chromatography with an ion exchange resin, gel filtration, electrophoresis, etc.

[0010] When the degree of purification is increased by such procedures, protein C or activated protein C is liable to become unstable. Even a product of lower purity also shows a decrease in activity due to procedures such as storage, freezing, lyophilization, heating, etc. The present invention is primarily aimed at the stabilization of such protein C or activated protein C which became unstable with the increase of purification degree. Protein C or activated protein C to be stabilized in accordance with the present invention may be either in the form of solution or powder.

[0011] In the stabilization method for protein C or activated protein C of the present invention, a salt containing as a stabilizing agent sodium ions at a concentration of preferably 50 mM to 200 mM, at least one amino acid and albumin are added to a buffer containing 100 to 2500 U/ml of protein C or activated protein C. The salt and amino acid may be used each alone or in a combination of two or more thereof. A Preferred buffer includes, for example, sodium citrate, sodium phosphate, and sodium sulfate, etc. The amino acid is added to reach a final concentration of 0.005 M to 0.1 M, more preferably 0.01 M to 0.05 M. Albumin is added to reach an appropriate concentration which may be determined based on a common sense or from the economical point of view, preferably to reach a concentration of 0.5% (W/V) to 10% (W/V). The unit "% (W/V)" as used herein denotes an amount of a solute dissolved in one liter of a solution, for example, when 10 g of a solute is dissolved in one liter of a solution, the concentration is 1% (W/V). In a preferred embodiment of the present invention, a non-ionic surfactant such as Tween 80 may optionally be added to reach a concentration of 0.0005% (W/V) to 0.1% (W/V) to accelerate the stabilization effect.

[0012] A typical embodiment of the present invention is an aqueous buffer solution containing protein C or activated protein C, which comprises 100 to 2500 U/ml of protein C and/or activated protein C, 50 to 200 mM of sodium ions, 5 to 100 mM of an amino acid, and further either one or a combination of 0.5 to 10% (W/V) of albumin and 0.0005 to 0.1% (W/V) of a non-ionic surfactant.

[0013] When a stabilizing agent is added to protein C or activated protein C in the form of powder, it is used in such an amount that the concentration of the stabilizing agent falls in the above-mentioned range when said powder is dissolved.

[0014] Thus, in another typical embodiment of the present invention, a composition containing protein C or activated protein C comprises 1×10^5 to 2.5×10^6 U of protein C and/or activated protein C, 50 to 200 mg equivalent weight of sodium ions, 5 to 100 millimoles of an amino acid, and further either one or a combination of 5 to 100 g of albumin and 0.005 to 1 g of a non-ionic surfactant.

[0015] The manner of addition of these ingredients may not be specified but includes various methods, for example, by adding directly powdery materials of the present invention to a buffer solution containing protein C or activated protein C; or by first dissolving said powdery materials in water or a suitable buffer and then adding the solution to the buffer solution containing protein C or activated protein C; or by mixing said powdery materials with a protein C- or activated protein C-containing powder. Addition may be carried out either during the process of isolation and purification of said protein or the process for producing a pharmaceutical preparation.

[0016] When a solution containing protein C or activated protein C added with the stabilizing agent of the present invention is stored, or subjected to procedures such as isolation and purification, or in a process for producing a pharmaceutical preparation in the state of solution, it is preferably done at 0 to 30°C, more preferably at 0 to 10°C. When said solution is stored in a frozen state, it is preferably done at a lower temperature than the freezing point, more preferably at lower than -20°C, or when it is stored in a lyophilized state, it is preferably done at room temperature or lower. By using the solution containing protein C or activated protein C containing the stabilizing agent of the present invention, the activity of protein C or activated protein C can be stably maintained even during storage in the state of a solution, or in a frozen or lyophilized state, or even in the treatment thereof such as isolation and purification or process for producing a pharmaceutical preparation.

[0017] The activity of APC was measured in accordance with the following procedures.

[0018] One unit of APC activity is defined as the amount of APC which prolongs twice an activated thromboplastin time (APTT; seconds) of normal human plasma. Accordingly, the activity of APC is measured wherein APTT in seconds is measured for normal human plasma to which a diluted sample is added and the dilution at which the measured APTT

value is twice that of the control (buffer) is determined and regarded as the activity of APC for samples.

(Procedures)

[0019] A sample is diluted with a veronal buffer containing 1% human serum albumin to, for example, 400 times, 500 times, 800 times of 1000 times dilution. To each 100 µl of either control (buffer) or samples of each dilution are added 100 µl of normal human plasma (e.g. Citrol I: Baxter Diagnostics Inc.) and 100 µl of APTT reagent (e.g. Actin: Baxter Diagnostics Inc.) at 37°C successively with an interval of 15 seconds, the mixture is stirred, and after 2 minutes, 100µl of 0.025 M $CaCl_2$ is added and the coagulation time is measured. (Calculation of activity)

[0020] A linear regression formula and a correlation coefficient of $10^3/X$ and Y are obtained from the APTT values (Y) at each dilution (X) of the control and the samples as follows:

$$Y = A\,(10^3/X) + B$$

[0021] A value of X_1 obtained from the following formula:

$$X_1 = 10^3\,\{(Y_1\text{-}B)/A\}$$

wherein Y_1 is a value twice that of the APTT (seconds) of control, is regarded as the activity of APC (U/ml) for samples.

[0022] The activity of protein C was measured by using "Staclot Protein C" manufactured by Boehringer Mannheim.

[0023] The present invention is illustrated in more detail by means of the following Examples but should not be construed to be limited thereto.

Example 1

Effect of various counterions on the stability of APC:

[0024] To a solution containing human activated protein C having an activity of 500 U/ml was added 2.5% human serum albumin (hereinafter also abbreviated as "HSA"). The solution was then dialyzed against a solution of Na citrate, Na phosphate and Na sulfate (each 20 mM), containing 0.7% NaCl and 0.067 M glycine. After dialysis, each solution was left to stand at 37°C for 24 hours and the activity was measured. The results are shown in Table 1. All the counterions tested, Na citrate, Na phosphate and Na sulfate, showed a similar satisfactory stabilizing effect.

Table 1

Effect of various counterions on the stability of APC	
Counterion	Rate of remaining activity (%) (37°C, 24 hours)
Na citrate 20 mM	97.4
Na phosphate 20 mM	94.8
Na sulfate 20 mM	92.2

Example 2

Effect of various amino acids on the stability of APC:

[0025] To a solution containing activated protein C having an activity of 500 U/ml was added 2.5% HSA. The solution was then dialyzed against a solution of sodium citrate buffer containing 0.7% NaCl and each 0.05 M of either glycine, alanine, lysine, arginine, aspartic acid or glutamic acid. After dialysis, each solution was left to stand at 37°C for 24 hours and the activity was measured. The results are shown in Table 2. All of the above six amino acids showed a high stabilizing effect without deteriorating the stability of APC.

Table 2

Effect of various amino acids on the stability of APC	
Amino acid (0.5%)	Rate of remaining activity (%) (37°C, 24 hours)
No amino acid	86.1
Gly	97.7
Ala	97.7
Lys	107
Arg	102
Asp	108
Glu	106

Example 3

Effect of HSA addition:

[0026] Solutions containing human activated protein C (1700 U/ml), 20 mM citrate, 0.7% NaCl and 0.067 M glycine with and without addition of 2.5% HSA were prepared. The solutions were then left to stand at 37°C and 4°C. The activity was measured over a period of time in accordance with the method described herein and a retention percent of activity was obtained. The results are shown in Table 3.

Table 3

Retention percent of activity under respective conditions (%)						
At 37°C:						
	Retention time (hour)					
	0	1	3	6	8	24
HSA (+)	100	-	99.2	-	101	97.4
HSA (-)	100	88.8	92.0	86.2	90.9	79.3
At 4°C:						
	Retention time (day)					
	0	1	3	5	7	14
HSA (+)	100	-	105	-	-	98.1
HSA (-)	100	112	99.1	104	98.9	85.2

[0027] As will be understood from the results shown in Table 3, the system without HSA can not prevent decrease in the activity of APC when it is left standing. From these results, it is concluded that 2.5% HSA extremely stabilizes APC.

Example 4

Effect of HSA on the stability of APC:

[0028] The data shown in Table 4 indicate that the activity of APC is dependent on the concentration of HSA. To a solution of activated protein C (500 U/ml) was added 0.5 to 10.0% HSA. This solution was put in a storage vessel and left to stand at 37°C. After 24 hours, a sample was taken and the activity was measured. In case of no addition of HSA,

the activity was decreased by about 20% whereas in case of addition of HSA at a concentration ranging from 05 to 10.0%, almost no decrease in the activity was observed and APC remained stable.

Table 4

Effect of HSA on the stability of APC activity	
HSA (%)	Rate of remaining activity (%) (37°C, 24 hours)
0.0	79.0
0.5	96.0
2.5	97.4
5.0	98.0
10.0	102

Example 5

Effect of Tween 80 on the stability of APC:

[0029] To a solution containing human activated protein C (500 U/ml), 20 mM citrate, 0.7% NaCl and 0.067 M glycine was added 0.0005% to 0.1% of a non-ionic surfactant, Tween 80 (trade name). This solution was put in a storage vessel and left to stand at 37°C. After 24 hours, a sample was taken and the activity was measured. The results are shown in Table 5. In case of no addition of Tween 80, the activity was decreased by about 20% and the stability was deteriorated whereas in case of addition of Tween 80 at a concentration ranging from 0.0005 to 0.1%, no change in the activity was found and the solution maintained a high stability.

Table 5

Tween 80 (%)	Rate of remaining activity (%) (37°C, 24 hours)
No addition	79
0.0005	101
0.025	104
0.1	103

Example 6

Effect of NaCl on the quality of lyophilized preparation:

[0030] An APC lyophilized preparation containing APC 500 U/ml, HSA 2.5%, Gly 0.067 M and Na citrate 20 mM was prepared such that it contains sodium chloride at a concentration ranging from 1 mM to 500 mM. Each lyophilized preparation was stored at 60°C for a month and the appearance quality of the solids was observed as shown in Table 6.

Table 6

Quality of lyophilized preparation containing NaCl at a selected concentration		
Na chloride (mM)	Quality of solids	
	Pre-storage	After One month storage at 60°C
1	White solid, shrunk	Extremely shrunk
10	White solid, a little shrunk	Same as pre-storage
50	White porous lump	Same as pre-storage
100	White porous lump	Same as pre-storage
500	Glassy shrunk lump	Same as pre-storage

[0031] As is clear from the results shown in Table 6, a solid APC preparation is difficult to formulate into a pharmaceutical preparation in the case of the addition of sodium chloride both at a higher and lower concentration. The presence of sodium chloride at a concentration of 50 mM to 200 mM is considered to contribute to the stabilization of the lyophilized preparation.

Example 7

APC activity in a lyophilized preparation:

[0032] A citrate buffer solution containing the stabilizing agent of the present invention (0.7% NaCl, 0.067 M glycine and 2.5% HSA) was prepared so that it contains an APC activity of 100 to 2500 U/vial and is divided into vials under sterile conditions, which were lyophilized and sealed. Each vial was left to stand at 10°C, 15°C and 60°C and the decrease in the activity was determined. The results are shown in Table 7. The data show that the method for stabilizing APC of the present invention is effective in the state of lyophilization.

Table 7

Rate of remaining activity (%)				
APC (U/vial)	Time	10°C	15°C	60°C
100	30 months	100	101	-
500	30 months	97	99	-
	11 days			99
1000	30 months	99	97	-
2500	30 months	98	95	-
	11 days			105

Example 8

Effect of repeated freezing-melting on the stability of APC:

[0033] To a citrate buffer solution containing activated protein C having an activity of 500 U/ml was added the stabilizing agent of the present invention (0.7% NaCl, 0.067 M glycine and 2.5% HSA). The resulting solution was subjected to repetition (5 times, 10 times, 15 times and 20 times) of freezing (-80°C) and melting and the activity was measured. The results are shown in Table 8. In spite of 20 repetitions of freezing-melting, the activity of APC showed no significant change and remained stable.

Table 8

Repetition	Rate of maintaining activity (%)
5	102
10	95.1
15	106
20	99.2

Claims

1. A method for stabilizing protein C or activated protein C which comprises adding, to a salt buffer containing protein C or activated protein C and sodium ions, at least one amino acid, and further either one or a combination of albumin and a non-ionic surfactant.

2. The method for stabilizing protein C or activated protein C of claim 1 wherein said amino acid is added to reach a final concentration of 0.005 M to 0.1 M.

3. The method for stabilizing protein C or activated protein C of claim 1 or 2 wherein said amino acid is selected from naturally occuring amino acids.

4. The method for stabilizing protein C or activated protein C of claim 3 wherein said amino acid protein is selected from glycine, alanine, lysine, arginine, aspartic acid and glutamic acid.

5. The method for stabilizing protein C or activated protein C of claim 1 wherein said albumin is added to reach a final concentration of 0.5% (W/V) to 10% (W/V).

6. The method for stabilizing protein C or activated protein C of claim 1 wherein said non-ionic surfactant is added to reach a final concentration of 0.0005% (W/V) to 0.1% (W/V).

7. An aqueous buffer solution containing protein C and/or activated protein C which contains 100 to 2500 U/ml of protein C and/or activated protein C, 50 to 200 mM of sodium ions, 5 to 100 mM of an amino acid, and further either one or a combination of 0.5 to 10% (W/V) of albumin and 0.0005 to 0.1% (W/V) of a non-ionic surfactant.

8. A composition containing protein C and/or activated protein C which contains 1×10^5 to 2.5×10^6 U of protein C and/or activated protein C, 50 to 200 mg equivalent weight of sodium ions, 5 to 100 millimoles of an amino acid, and further either one or a combination of 5 to 100 g of albumin and 0.005 to 1 g of a non-ionic surfactant.

Patentansprüche

1. Verfahren zum Stabilisieren von Protein C oder aktiviertem Protein C, umfassend Zugeben zu einem Salzpuffer, enthaltend Protein C oder aktiviertes Protein C und Natriumionen, von mindestens einer Aminosäure und weiter von Albumin oder einem nicht-ionischen Tensid oder einer Kombination davon.

2. Verfahren zur Stabilisierung von Protein C oder aktiviertem Protein C nach Anspruch 1, wobei die Aminosäure so zugegeben wird, dass eine Endkonzentration von 0,005 M bis 0,1 M erreicht wird.

3. Verfahren zur Stabilisierung von Protein C oder aktiviertem Protein C nach Anspruch 1 oder 2, wobei die Aminosäure aus natürlich vorkommenden Aminosäuren ausgewählt ist.

4. Verfahren zur Stabilisierung von Protein C oder aktiviertem Protein C nach Anspruch 3, wobei die Aminosäure aus Glycin, Alanin, Lysin, Arginin, Asparaginsäure und Glutaminsäure ausgewählt ist.

5. Verfahren zur Stabilisierung von Protein C oder aktiviertem Protein C nach Anspruch 1, wobei das Albumin so

zugesetzt wird, dass eine Endkonzentration von 0,5% (W/V) bis 10% (W/V) erhalten wird.

6. Verfahren zur Stabilisierung von Protein C oder aktiviertem Protein C nach Anspruch 1, wobei das nicht-ionische Tensid so zugegeben wird, dass eine Endkonzentration von 0,0005% (W/V) bis 0,1% (W/V) erhalten wird.

7. Wässrige Pufferlösung, enthaltend Protein C und/oder aktiviertes Protein C, die 100 bis 2500 U/ml Protein C und/oder aktiviertes Protein C, 50 bis 200 mM Natriumionen, 5 bis 100 mM einer Aminosäure und weiter 0,5 bis 10% (W/V) Albumin oder 0,0005 bis 0,1% (W/V) eines nicht -ionischen Tensids oder eine Kombination davon enthält.

8. Zusammensetzung, enthaltend Protein C und/oder aktiviertes Protein C, die 1×10^5 bis 2×10^6 U an Protein C und/oder aktiviertem Protein C, 50 bis 200 mg Äquivalentgewicht Natriumionen, 5 bis 100 mM einer Aminosäure und weiter 5 bis 100 g Albumin oder 0,005 bis 1 g eines nicht-ionischen Tensids oder eine Kombination davon enthält.

Revendications

1. Procédé pour stabiliser la protéine C ou la protéine C activée qui comprend l'addition, à un tampon constitué par un sel contenant de la protéine C ou de la protéine C activée et des ions sodium, d'au moins un acide aminé et de plus soit d'une combinaison d'albumine et d'un tensioactif non ionique soit d'un seul de ces deux constituants.

2. Procédé pour stabiliser la protéine C ou la protéine C activée selon la revendication 1, dans lequel ledit acide aminé est ajouté pour atteindre une concentration finale comprise entre 0,005 M et 0,1 M.

3. Procédé pour stabiliser la protéine C ou la protéine C activée selon la revendication 1 ou 2, dans lequel ledit acide aminé est choisi parmi les acides aminés d'origine naturelle.

4. Procédé pour stabiliser la protéine C ou la protéine C activée selon la revendication 3, dans lequel ledit acide aminé est choisi parmi la glycine, l'alanine, la lysine, l'arginine, l'acide aspartique et l'acide glutamique.

5. Procédé pour stabiliser la protéine C ou la protéine C activée selon la revendication 1, dans lequel ladite albumine est ajoutée pour atteindre une concentration finale comprise entre 0,5% (m/v) et 10% (m/v).

6. Procédé pour stabiliser la protéine C ou la protéine C activée selon la revendication 1, dans lequel ledit tensioactif non ionique est ajouté pour atteindre une concentration finale comprise entre 0,0005% (m/v) et 0,1% (m/v).

7. Solution aqueuse tampon contenant de la protéine C et/ou de la protéine C activée, qui contient de 100 à 2500 U/ml de protéine C et/ou de protéine C activée, de 50 à 200 mM d'ions sodium, de 5 à 100 mM d'un acide aminé et de plus soit une combinaison de 0,5 à 10% (m/v) d'albumine et de 0,0005 à 0,1% (m/v) d'un tensioactif non ionique soit un seul de ces deux constituants.

8. Composition contenant de la protéine C et/ou de la protéine C activée qui contient de 1×10^5 à $2,5 \times 10^6$ U de protéine C et/ou de protéine C activée, de 50 à 200 mg d'équivalents en masse d'ions sodium, de 5 à 100 millimoles d'un acide aminé et de plus soit une combinaison de 5 à 100 g d'albumine et de 0,005 à 1 g d'un tensioactif non ionique soit un seul de ces deux constituants.